รายงานวิจัยฉบับสมบูรณ์

โครงการ การยับยั้งการทำงานเอนไซม์โคลิเนสเทอเรสในระบบไหลเวียนของเลือดและในส่วนของสมองส่วนฮิพโพ-แคมพัสในสัตว์ทดลองจากผลของสารสกัดจากต้นพุดพิทยา (ทาเบอร์นามอนทานาไดวาริคาทา)

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สัญญา เลขที่ RMU4880013

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สนับสนุนโดยทบวงมหาวิทยาลัยและสำนักงานกองทุนสนับสนุนการวิจัย

คำนำ

ผู้วิจัยขอขอบคุณ รศ. ดร. กรกนก อิงคนินันท์ในการช่วยจัดทำสารสกัดจากต้นพุดพิทยา (ทาเบอร์นามอนทานาไดวาริคาทา) ขอขอบคุณ อาจารย์วาสนา ปรัชญาสกุล และ นายอนุชา พงศ์พันธ์ภารดร บัณฑิตปริญญาโท ในฐานะผู้ช่วยวิจัยที่ช่วยดำเนินการทดลองและให้ความช่วยเหลือในทุกทางตลอดโครงการ นอกจากนี้ ขอขอบคุณศูนย์วิจัย Cardiac Electrophysiology Research and Training Center ภายใต้การดูแลของ รศ. นพ. ดร. นิพนธ์ ฉัตรทิพากร คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ที่ให้ความอนุเคราะห์ในการใช้เครื่องมือสำหรับงานวิจัยครั้งนี้ การศึกษานี้ได้รับการสนับสนุนจากกองทุนวิจัยจาก Thailand Research Fund (RMU4880013)

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สารบัญ

สารบัญรูปภาพ

บทคัดย่อภาษาไทย

แนวทางการรักษาโรคอัลไซเมอร์ (Alzheimer's disease AD) ซึ่งเป็นโรคที่เกิดจากความผิดปกติของการทำงานของระบบประสาท cholinergic system ในปัจจุบัน คือการใช้สารที่ไปยับยั้งการทำงานของเอนไซม์ acetylcholinesterase เพื่อเพิ่มการทำงานของระบบ cholinergic system ในการศึกษาในหลอดทดลอง ก่อนหน้านี้พบว่าสารสกัดจากต้นพุดพิทยาหรือพุดซ้อน (*Tabernaemontana divaricata* extract, TDE) หนึ่งในสมุนไพรในตำรายาอายุวัฒนะ ที่ความเข้มข้น 0.1 mg/ml สามารถยับยั้งการทำงานของเอนไซม์ acetylcholinesterase ได้อย่างมีประสิทธิภาพมากกว่า 90% เมื่อเทียบกับกลุ่มควบคุมอย่างไรก็ตามผลการยับยั้งการทำงานของเอนไซม์ acetylcholinesterase และผลของสารสกัดนี้ ต่อการทำงานของระบบประสาทในสมองโดยเฉพาะสมองส่วน cerebral cortex และ hippocampus ซึ่งเป็นสมองที่มีบทบาทสำคัญในการจำและการเรียนรู้ยังไม่มีการทำการศึกษาในสัตว์ทดลองมาก่อนเลย ดังนั้นในการศึกษานี้จึงศึกษาถึงผลของสารสกัดจากต้นพุดพิทยาต่อ การทำงานของสมองส่วน cerebral cortex และ hippocampus และการทำงานของเอนไซม์ acetylcholinesterase ในสัตว์ทดลอง โดยใช้เทคนิคการย้อมติดโปรตีนที่ชื่อ *c-fos* ซึ่งโปรตีนนี้จะเป็นตัวชี้วัดว่าเซลล์ประสาทมีการถูกกระตุ้นให้เกิดการทำงานขึ้นหรือไม่ โดยเราศึกษาในสมองส่วน cerebral cortex และใช้วิธี Ellman's colorimetric ในการศึกษาถึงการทำงานของเอนไซม์ acetylcholinesterase ทั้งในสมองส่วน cerebral cortex และในเลือด ของหนูขาวพันธ์ Wistar โดยจะแบ่งหนูออกเป็น 4 กลุ่ม คือ หนูที่ได้รับสารสกัดต้นพุดพิทยาในขนาด 0, 250, 500 และ 1,000 mg/kg ตามลำดับ จากการศึกษาพบว่า สารสกัดต้นพุดพิทยาสามารถเพิ่มการทำงานของเซลล์ประสาท และสามารถยับยั้งการทำงานของเอนไซม์ acetylcholinesterase ในสมองส่วน cerebral cortex ได้เช่นเดียวกับการศึกษาในหลอดทดลอง จากนั้นเราได้ศึกษาถึงคุณสมบัติทางสรีรวิทยาของเซลล์ประสาทในสมอง hippocampus ซึ่งเป็นสมองอีกส่วนหนึ่งที่บทบาทสำคัญเกี่ยวกับความจำและการเรียนรู้ โดยการศึกษานี้ได้ใช้ extracellular recording เพื่อศึกษาถึงการเปลี่ยนแปลงของ field excitatory postsynaptic potential (fEPSPs) ซึ่งจัดเป็นตัวชี้วัดถึงการทำงานปกติของการสื่อประสาทระหว่างเซลล์ประสาท และจากการศึกษาพบว่า สารสกัดต้นพุดพิทยามีผลลด field excitatory postsynaptic potential (fEPSPs) ของสมองส่วน CA1 hippocampus โดยการลดลงของ fEPSPs ที่พบจะถูกยับยั้งโดยการให้ยา atropine แต่ไม่ถูกยับยั้งโดยยา pancuronium นอกจากนี้ฤทธิ์การยับยั้ง fEPSPs นี้ยังมีผลเหมือนกับผลการให้สาร acetylcholine หรือการให้ยา galanthamine ซึ่งจัดเป็นยาที่มีคุณสมบัติ AChE-Is ที่ใช้รักษาในผู้ป่วย AD จากผลการศึกษาทั้งหมดสามารถสรุปได้ว่าสารสกัดต้นพุดพิทยาออกฤทธิ์เป็นตัวยับยั้งการทำงานของเอนไซม์ acetylcholinesterase ในสมอง โดยสารสกัดต้นพุดพิทยาจะมีผลไปยับยั้งการทำงานของเอนไซม์ acetylcholinesterase ในสมองส่วน cortex และยังสามารถเพิ่มการทำงานของระบบประสาทในสมองส่วน cortex และออกฤทธิ์ควบคุม synaptic transmission ซึ่งฤทธิ์ที่เกิดขึ้นนี้จะคล้ายกับการออกฤทธิ์ของสารที่ยับยั้งการทำงานเอนไซม์ acetylcholinesterase ที่

เป็นที่รู้จักทั่วไปคือ galanthamine ดังนั้น สารสกัดจากต้นพุดพิทยานี้น่าจะมีประโยชน์ในการศึกษาหาส่วนประกอบสำคัญในสารสกัดนี้ เพื่อใช้ในการรักษาโรคอัลไซเมอร์ ในอนาคต

ABSTRACT

The current pharmacotherapy for Alzheimer's disease (AD), a progressive neurodegenerative disorder of cholinergic system, is the use of acetylcholinesterase (AChE) inhibitors. A previous *in vitro* study showed that *Tabernaemontana divaricata* extract (TDE) at 0.1 mg/ml inhibited more than 90% of AChE activity. However, neither the acetylcholinesterase (AChE) inhibitory effects nor the effect on neuronal activity in cerebral cortex, a brain region critical for learning and memory, of TDE has been investigated *in vivo*. To investigate the effects of TDE on cortical neuronal activity as well as AChE activity in animal models, we used *c-fos* immunohistochemical techniques to determine the neuronal activity in cerebral cortex and the Ellman's colorimetric method to investigate the cortical and circulating cholinesterase (ChE) activity in Wistar rats following the acute administration of TDE at various doses (0, 250, 500 and 1000 mg/kg). We found that TDE enhanced neuronal activity in cerebral cortex and inhibited cortical AChE activity. To further investigate the physiological properties of TDE in slices of hippocampus, the other brain region in learning and memory, we used extracellular recordings of field potential, and found that TDE significantly reduced field excitatory postsynaptic potentials (fEPSPs). The reduction in potential was prevented by atropine, but not pancuronium, and it was not accompanied by evident changes in the EPSP waveform. The modulation of synaptic function with TDE was similar to the effect of exogenous acetylcholine (ACh) and galanthamine. Our findings describe novel characteristics of TDE as an AChE inhibitor in the brain. The effects of TDE on the inhibition of cortical AChE activity, the enhancement of cortical neuronal activity as well as the effect on the synaptic transmission in hippocampus are similar to other well-known AChE-I agents galanthamine. These findings suggest that TDE could be beneficial as a possible novel therapeutic agent for AD in the future.

EXECUTIVE SUMMARY

1. ความสำคัญและที่มาของปัญหา

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease with many cognitive and neuropsychiatric manifestations that result in progressive disability. Neuropathologically, AD is characterized by the appearance of neuritic plaques and neurofibrillary tangles in the brain; these changes are paralleled behaviorally by impairments in cognitive function [1-3]. The cholinergic system seems to be the predominantly affected neurotransmitter system in this disease[4]. Cholinergic innervation of hippocampus is required for some forms of learning and memory. The degeneration of cholinergic afferents to hippocampus from the medial septum is one hallmark of AD. In fact, the severity of cognitive decline is correlated with cholinergic impairment[5]. Thus the overlapping connections between acetylcholine (ACh), hippocampal synaptic plasticity, learning and memory, and AD motivate investigators to study mechanisms by which ACh and its receptors modulate hippocampal function, since alterations in these mechanisms may underlie some cognitive deficiencies in AD. The currently best available pharmacotherapy for AD is the use of acetylcholinesterase (AChE) inhibitors (AChE-Is), which indirectly elevate the attenuated acetylcholine-concentrations in the AD brain, thereby enhancing cholinergic function. Several promising AChE-Is such as tacrine[6;7], donepezil[8], rivastigmine[9] and galanthamine[10] have been developed. However, the drug of choice for the treatment of AD has not been established. Thus, the search for other new AChE inhibitors is of interest to investigators

Tabernaemontana divaricata (TD) has been reported to be a rich source of various alkaloids[11]. It is one of 32 plant materials used in the traditional rejuvenating remedies in Thailand. In Thai herbal medicine, these remedies are believed to improve memory. In addition, native people in America, Africa and Continental Asia have used this plant as a central nervous system stimulant. However, there have been very few scientific studies to support the idea that TD can improve memory. Recently, Ingkaninan et al (2003) demonstrated that ethanolic extracts of TD (0.1 mg/ml) inhibit more than 90% of AChE activity *in vitro* study [12]. Therefore, the TD extracts could also have a therapeutic benefit in the treatment of AD. Nevertheless, the AChE inhibitory effects as well as the effect of increasing neuronal activity in hippocampus, a brain region critical

for learning and memory, from TD extracts have not investigated in an *in vivo* animal model.

The hypothesis of this proposal is that TD extracts can 1) inhibit both circulating and neuronal acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activity in normal rats and 2) increase neuronal activity in hippocampus.

2. วัตถุประสงค์

To test these hypotheses, this proposal will investigate the following specific aims:

***Specific Aim 1*: To test the hypothesis that Tabernaemontanan divaricata (TD) extracts can inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activity in the normal rats.** We will use the colorimetric method originally described by Ellman et al 1961[13] to determine:

1.1: Whether acute administration of TD extracts at various doses (low to high) into the rats at the specific time point can inhibit circulating and/or hippocampal AChE and BuChE activity compared to the control group.

1.2: Whether the inhibitory effects of TD extracts at the maximally effective doses (as determined in specific aim 1.1) on circulating and hippocampal AChE and BuChE activity vary when TD is administrated at different time points.

***Specific Aim 2*: To investigate whether TD extracts can enhance neuronal activity in hippocampus**. Our preliminary data showed that TD extracts can inhibit AChE activity in the circulation and hippocampus as shown in Figure 2 and Table 2 in the preliminary data section, suggesting that TD extracts could affect the central nervous system. We will also use *c-fos*-immunoreactivity as a neuronal marker to test whether TD extracts can increase neuronal activity in the hippocampal region.

Specific Aim 3: **To test the hypothesis that TD extracts enhance synaptic transmission in hippocampus via activation of acetylcholine receptors.** We will use a combination of whole-cell voltage-clamp recordings, extracellular dendritic field potential recordings and extracellular popspike recordings as well as pharmacological tools in acutely prepared brain slices to determine:

3.1 Whether TD extracts can increase neuronal excitatory synaptic transmission in hippocampal circuits.

3.2. If so, whether the enhancing effect of TD is mediated by activation of acetylcholine receptors.

3. ระเบียบวิธีการวิจัย

Experimental Design

General Methods

Animal Care

400 male Wistar rats, weighing 100-150 g, will be purchased from the National Animal center, Salaya campus, Mahidol University, Bangkok. All rats are housed two or three per cage with free access to food and water for at least 1 week prior to the study. The research protocol will adhere to the “Guide for the Care and Use of Animals” and follow appropriate Chiang Mai University Standard Operating Procedure for animal identification, housing and diet prior to experiments.

Extract of TD

The plant materials will be cut into small pieces and dried in a hot air oven at 55°C. The dried materials will be macerated in ethanol for 3 days and filtered. The filtrate will be evaporated under reduced pressure until dryness. The residue from the filtration will be macerated in ethanol again for 3 days and filtered. The filtrate will be evaporated with the same procedure and combined with the extract from the first extraction. Each lot of TD extracts will be analyzed for AChE inhibitory activity *in vitro*.

To confirm the quality of TD extracts in each experiment, each lot of TD extracts will be microplate assayed for AChE activity in vitro before use in vivo

The assay for measuring AChE activity will be modified from the assay described by Ellman et al. (1961)[13] and Ingkaninan et al. (2003)[12]. Briefly, 125 µl of 3 mM DTNB, 25 µL of 15 mM ATCI, 50 µl of buffer, and 25 µl of sample dissolved in buffer containing not more than 10% methanol will be added to microplate wells, followed by 25 µl of 0.28 U/ml AChE. The microplate will then be read at 405 nm every 5 sec for 2 min by a CERES UV 900C microplate reader (Bio-Tek instrument, USA). The velocities of the reactions will be measured. Enzyme activity will be calculated as a percentage of the velocities compared to that of the blank. Inhibitory activity will be calculated by subtracting the percentage if enzyme activity from one hundred percent. Every experiment will be done in triplicate. Stock solutions of samples in Tris-HCl buffer containing not more than 10% MeOH will be diluted serially with Tris-HCl Buffer to obtain 8-9 different concentrations. The IC_{50} value, corresponding to the inhibitor

concentration that caused 50% inhibitory activity, will be analyzed using the software package Prism (Graph Pad Inc, San Diego, USA).

ChE activity determinations in blood and hippocampal samples

Determination of ChE will be based on the colorimetric method originally described by Ellman et al (1961)[13], adapted for determining the enzyme activity in rat blood and hippocampal homogenates.

The fresh hippocampal tissue will be weighed and then homogenized in 10 parts of 0.1 M phosphate buffer pH 7.4, which contains 1% Triton-X 100. Following centrifugation at 15,000 rpm for 15 minutes at 4^{o}C, the clear supernatants will be removed and serve as the enzyme source. ChE activity will be determined in 50 µl aliquots of RBC or the hippocampal homogenates (run as duplicates). The reaction will be started by adding 1) 0.5 mM acetylthiocholine-iodide (ASCh), the commonly used substrate for *in vitro* AChE determinations (Ellman et al, 1961)[13] or 2) 0.5 mM butyrylthiocholine (BuCh) iodide, the commonly used substrate for *in vitro* BuChE determinations, and 0.25 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DNTB), both dissolved in phosphate buffer pH7.4 (0.1M), The plate will be then immediately placed into the automatic Microreader and the yellow reaction product will be quantified at 22^{o}C using 450 nm as the wave-length. The reaction will be monitored over a period of 10 min with readouts taken every 10s, which will then be processed by a program controlled by the plate reader and stored on a computer, Quantification of the enzymatic activity will be based on a change in optical density in the linear-range over time using the molar extinction coefficient of the reaction products. The spectophotometric absorption will be quantitatively measured and expressed as nmol acetylcholine hydrolysed/min/ml RBC or mg tissue and nmol butylcholine hydrolysed/min/ml plasma or mg tissue.

Data analysis of ChE activity

Data will be expressed throughout as means$\pm$SEM. Statistical analyses will be carried out using ANOVA and post-hoct analyse to calculate the significance effects in both TD treated and control rats.

Fos immunohistochemistry

Two hours after the TD injections, animals will be deeply anesthetized with pentobarbital (80 mg/kg) and perfused intracardially with PBS, followed by 4% paraformaldehyde. The hippocampus will be removed, postfixed and placed in 30% sucrose overnight and then frozen and cut into 40-µm-thick, transverse frozen sections. Every section will be processed for Fos, using a rabbit polyclonal antibody (Santa Cruz,

1: 1,000 dilution). A black reaction product will be produced with a standard ABC reaction (Vectastatin Elite kit; Vector Labs) with nickel intensification using Vectastain, and sections will be mounted on slides. Processing for Fos will be similar to that described previously[37].

Data analysis of Fos immunohistochemistry

Changes in the number of *c-fos*-positive cells in the hippocampal region between different treatment groups will be counted with a double blind technique. Data will be expressed throughout as means$\pm$SEM. Statistical analyses will be carried out using ANOVA to calculate significance for both TD treated and control rats.

Electrophysiological Studies

Preparation of brain slices

Coronal hippocampal slices (400 μm) will be prepared using a vibratome from Wistar rats 4-5 weeks old and 100-150 g weight, which will be similar in age and weight to those used to the determine ChE activity. Standard methods of brain slice preparation will be employed[31]. The solution used for slice preparation and recording is a standard aCSF solution containing (in mM): NaCl 119, KCl 2.5, $CaCl_2$ 2.5, $MgSO_4$ 1.3, NaH_2PO_4 1, $NaHCO_3$ 26, glucose 10, saturated with 95%O_2 /5%CO_2 (pH 7.4). Submersion chambers will be used for storing slices and performing recordings. During recordings, slices will be continuously perfused at 3-4 ml/min with aCSF warmed to 28-30^{o}C.

Whole cell recordings

Experiments will focus on cells in the CA1 pyramidal cell layer, and will be conducted using the patch clamp technique as modified for blind recording in slices. Patch pipettes, pulled from borosilicate glass capillaries on a Sutter puller, will have resistances 3 - 5 mΩ For voltage clamp recording pipettes will be filled with a solution consisting of (in mM): CsCl, 110; CsOH, 25; EGTA, 11; CaCl2, 1; MgCl2, 2; and N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (HEPES), 10. The pH will be adjusted to 7.2 with CsOH. Only recordings with series resistances, in the range of 5-8 MΩ will be. For current clamp recordings, pipettes will be filled with a solution consisting of (in mM): K-gluconate, 135; KCl, 6; MgCl2, 2; HEPES, 10. The pH will be adjusted to 7.2 with KOH.

An Axopatch 200 (Axon Instruments) will be used to amplify current/voltage signals and the output will be continuously monitored on an oscilloscope and Gould chart recorder. Data will be filtered at 3 kHz, digitized at 10 kHz, and stored on a computer using

software written in Digitdata (Axon Instruments).

Extracellular recordings

To examine the net effects of glycine on the granule cell population, extracellular recordings of population spikes will be performed. In these experiments, bipolar stimulating electrode will be placed in the Schaffer' collaterals and a recording electrode (filled with 2 mM NaCl) will be placed in the CA1 pyramidal cell layer.

Data analysis of electrophysiology

Data from all experiments will be analyzed off-line. Popspike and evoked synaptic currents or potentials will be analyzed using software written in Digitdata. Spontaneous currents will be analyzed using MINI. Data will be expressed throughout as means±SEM. Differences between inter-event intervals and amplitude in control aCSF and following TD application will be determined using Student's paired t-test.

Experimental Design

Experimental Design for specific aim 1

Specific Aim1: **To test the hypothesis that Tabernaemontanan divaricata (TD) extracts can inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activity in normal rats.**

***Experiment protocol I*:** To determine whether acute administration of TD extracts of various doses administrated to rats at the specific time point can inhibit circulating as well as hippocampal AChE and BuChE activity compared to control rats.

Rationale: An *in vitro* study has shown that the ethanolic extracts from the TD plant at concentration of 0.1 mg/ml inhibit more than 90% of AChE activity by screening the AChE inhibitory activity, using Ellman's colorimetric method[12]. To determine whether TD extracts can similarly inhibit ChE activity in the circulating blood and hippocampus *in vivo*, different doses of TD extracts will be used in this study. Each dose of TD extracts will be studied for its pharmacological activities similarly to previous observational (hippocratic) screening reports[29]. The maximal doses of TD extract used will be 1 g/kg since this dose has been previously reported to cause 33% death[29].

Methods: Experiments will be performed on adult male Wistar rats. The rats will be divided into 5 groups (n=10 per group). Each group will be injected intraperitoneally with either 250 mg/kg (group1) or 500 mg/kg (group2) or 1 g/kg (group 3) of TD extracts dissolved in 100% ethanol (EtOH). Control animals (group 4) will be also intraperitoneally injected with EtOH and positive control animals (group 5) will be intraperitoneally injected with 10 mg/kg galanthamine (a AChE-I), since previous studies

indicate that 10 mg/kg can cause >30% AChE inhibition[38;39] . Each rat will be allowed to survive for two hours after injection of TD extracts before studying the ChE activity, since other ChE-Is exhibit maximal ChE inhibitory activity after two hours[40]. Then all rats will be sacrificed and blood and hippocampus will be collected for ChE activity determinations.

Experiment protocol II:- To determine whether the acute administration of TD extracts at the dose which exhibit the maximal ChE inhibitory effects (data from specific aim 1.1) differentially inhibit circulating and hippocampal AChE and BuChE activity in different time points.

Rationale: Preliminary data demonstrate that a single injection of TD extracts (500 and 1000 mg/kg) significantly inhibits AChE in both circulating blood and hippocampus in 2 hours (as shown in Figure 2 and Table 2). To determine the time course of the ChE inhibitory effect of TD, we will divide animals into 16 groups (n=10 per group). The 16 groups will be divided into 8 experimental groups (which will receive TD extracts in 100% ethanol vehicle) and 8 control groups (which will receive vehicle alone). The details of each experimental group are presented in Table 1. We will use time points selected according to the method described by Robichaud and Malone[41]. Following each time points, animals will be sacrificed and blood and hippocampi will be collected for ChE activity determinations.

Table 1: The details for experimental protocol

Time after drug administration (minutes)	A single dose of 100% EtOH	A single dose of 500 mg/kg TD in 100% EtOH
0	Group 1	Group 9
5	Group 2	Group 10
10	Group 3	Group 11
15	Group 4	Group 12
30	Group 5	Group 13
60	Group 6	Group 14
120	Group 7	Group 15
240	Group 8	Group 16

Experimental Design for specific aim 2

Specific Aim 2: **To investigate whether TD extracts can enhance neuronal activity in normal hippocampus**.

Experiment protocol III:- To determine whether TD extracts can increase neuronal activity in normal rats' hippocampus [a brain region important for learning and memory] by using *c-fos*-immunoreactivity as a marker for neuronal activity.

Rationale: Our preliminary data demonstrate that hippocampal AChE activity in animals injected with 500 or 1000 mg/kg of TD extracts dissolved in EtOH was less than that in animals that received EtOH injection alone, as shown in Figure 2. If TD extracts cause increased ACh level, they could also increase neuronal activity in the hippocampus. To further investigate whether TD extracts can increase hippocampal neuronal activity, we used *c-fos*-immunoreactivity as a marker for neuronal activity to compare the Fos-positive neurons between two treatment groups, which received either: (1) EtOH or (2) TD extracts dissolved in EtOH. Several studies have found a correlation between learning processes and immediate early gene (IEG) expression, particularly *c-fos*,[42;43]. The induction of proto-oncogenes or IEGs, i.e. *c-fos*, is rapid, transient and protein synthesis independent[44-46]. The protein products of IEGs are generated in the cell membrane, cytosol and importantly in the nucleus; thus they are ideally located for the regulation of gene expression. In normal physiological states, the basal level of IEG expression in the brain is low. However, different stimuli can induce IEG in neurons of CNS structures known to be involved in the processing of these stimuli. The analysis of IEG induction is a useful tool for investigating activated neuronal populations. The induction of *c-fos* protein after learning in certain neurons, i.e., hippocampal regions, can serve to identify these neurons as being involved in learning-associated signal processing. In this study, *c-fos* expression will be used to detect the earliest hippocampal regions involved in the TD-enhanced neuronal activity. Because *c-fos* is a cellular marker, the pattern of its staining can provide clues about the involvement of specific neuronal populations. In our preliminary experiments, animals injected with TD extracts dissolved in EtOH appear more alert than those that receive an EtOH injection alone. To address the possibility that TD extracts improve memory, we will determine the expression of *c-fos proteins* in hippocampal neurons. If *c-fos* expression following treatment with TD extracts dissolved in EtOH is greater than that observed following treatment with EtOH alone, it is possible that TD extracts can improve neuronal activity in brain regions responsible for learning and memory.

Methods: We will study *c-fos* expression in rat hippocampus following the acute treatment with (1) EtOH (as control group) and (2) 3 different doses of TD extracts dissolved in EtOH: 250 mg/kg, 500mg/kg and 1 g/kg intraperitoneal injection. Rats will be divided into 4 groups (n=10 per group). Each group will be injected intraperitoneally with either 250 mg/kg (group1) or 500 mg/kg (group2) or 1 g/kg (group 3) of TD extracts dissolved in 100% ethanol (EtOH) or 100% EtOH only (control group 4). Rats will be allowed to survive for 2 hours after injection and will then be sacrificed for *c-fos* immunohistochemical analysis. Two hours has been shown in several studies to be the time point of maximal *c-fos* expression after external stimulation[37].

Experimental Design for specific aim 3

***Specific Aim 3*: To test the hypothesis that TD extracts can enhance synaptic transmission in hippocampal regions via activation of acetylcholine receptors.**

Experimental protocol IV: We will use a combination of whole-cell voltage-clamp recordings, extracellular dendritic field potential recordings and extracellular popspike recordings in acutely prepared brain slices to determine whether TD extracts can increase neuronal excitatory synaptic transmission in hippocampal circuits. We will use a combination of whole-cell voltage-clamp recordings, extracellular dendritic field potential recordings and extracellular popspike recordings along with pharmacological tools (atropine, a cholinergic receptor antagonist) in acutely prepared brain slices to investigate whether the enhancing effect of TD is mediated via activation of acetylcholine receptors.

Rationale: Our preliminary data demonstrate that the number of Fos-positive neurons significantly increase after TD (1000 mg/kg) administration. We will further investigate whether TD can alter intrinsic firing properties and synaptic transmission in hippocampal circuits similarly to well-known AChE-I drugs such as galanthamine and, if so, whether these effects are mediated via activation of acetylcholine receptors.

Methods: Population spike and whole-cell voltage or current clamp recordings of CA1 pyramidal neurons in the presence or absence of TD will be performed. Bicuculline will be used in all experiments to isolate glutamatergic transmission. The effects of TD on glutamatergic transmission in rat hippocampal slices will be investigate for time and dose-dependence. Atropine will be used in some experiments to confirm that TD effects are mediated through acetylcholine receptors. Bath application of 10 μM ACh or 1 μM galanthamine [47] will be used as positive controls for this experimental protocol.

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4. แผนการดำเนินงานวิจัยตลอดโครงการในแต่ละช่วง 6 เดือน

ระยะเวลา	1-6 เดือน	7-12 เดือน	13-18 เดือน	19-24 เดือน	25-30 เดือน	31-36 เดือน
สั่งซื้อวัสดุและสกัดสาร TD	←					
ChE activity in specific aim 1	←	→				
Fos-immunohistochemistry in specific aim 2			←	→		
Electrophysiology in specific aim 3				←	→	
Data analysis			←	→	→	
Preparation for publication			←		→	→

5. ผลงาน/หัวข้อเรื่องที่คาดว่าจะตีพิมพ์ในวารสารวิชาการระดับนานาชาติในแต่ละปี
ชื่อวารสารที่คาดว่าจะตีพิมพ์:

1. The inhibition of cholinesterase activity in the in vivo study following the administration of crude alkaloidal fraction from root of Tabernaemontana Divaricata: Expected Journal: Journal of Ethnopharmacology

2. Crude extract of Tabernaemontana Divaricata enhances glutamatergic transmission in hippocampal CA1 neurons: Expected Journal: Brain Research

เนื้อหางานวิจัย

บทที่ 1: *TABERNAEMONTANA DIVARICATA* EXTRACT INHIBITS NEURONAL ACETYLCHOLINESTERASE ACTIVITY IN RATS.

บทนำ

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease, having many cognitive and neuropsychiatric manifestations that result in progressive disability. Neuropathologically, AD is characterized by the appearance of neuritic plaques and neurofibrillary tangles in the brain. These changes are paralleled behaviorally by impairments in cognitive function (Andreasen and Blennow, 2002; Blennow et al., 2001; DeKosky, 2003; Koo, 2002; Parihar and Hemnani, 2004; Russo et al., 2005). The cholinergic system has been shwon to be the predominantly affected neurotransmitter system in this disease (Giacobini, 2003). It has been shown that cholinergic innervation of cerebral cortex is required for some forms of learning and memory (Bigl et al., 1982). The degeneration of cholinergic afferents to cerebral cortex from the medial septum is one hallmark of AD (Coyle et al., 1983). In fact, the severity of cognitive decline is correlated with cholinergic impairment (Perry et al., 1978; Terry, Jr. and Buccafusco, 2003; Perry et al., 1999). Currently, the most effective available pharmacotherapy for AD is the use of acetylcholinesterase inhibitors (AChE-Is), which indirectly elevate the attenuated acetylcholine concentrations in the AD-affected brain, thereby enhancing cholinergic function (Barnes et al., 2000; Bickel et al., 1991; Disterhoft and Matthew, 2003; Giacobini, 2003; Liston et al., 2004). Although the use of AChE-Is (e.g., donezepil, rivastigmine and galantamine), a symptomatic pharmacological treatment of AD, has been shown to be beneficial on cognitive, functional and behavioral symptoms of the disease, it also causes undesired side effects (Bickel et al., 1991; Liston et al., 2004; Sweeney et al., 1989; Woodruff-Pak et al., 2001; Zarotsky et al., 2003). The most common adverse effects, related to cholinergic stimulation in the brain and peripheral tissues, include gastrointestinal, cardiorespiratory, extrapyramidal, genitourinary, and musculoskeletal symptoms, as well as sleep disturbances (Thompson et al., 2004). Therefore, the search for new AChE-Is, particularly from natural products, with higher efficacy and fewer side effects has been extensively investigated.

Tabernaemontana divaricata (*T. divaricata*) (L.) is a common garden plant in Southeast Asia and other tropical countries. It has been reported to be a rich source of various alkaloids, with various pharmacological properties (Van Beek et al., 1984). It has been used in traditional rejuvenation remedies in Thailand (Ingkaninan et al., 2003). In Thai herbal medicine, these remedies are believed to improve memory. In addition, native people in America, Africa and Continental Asia have used this plant as a central nervous system stimulant (Taesotikul et al., 1998). Despite its long-time use, there have been very few scientific studies to explain how *T. divaricata* can improve memory (Ingkaninan et al., 2003). Recently, Ingkaninan et al (2003) demonstrated that methanolic extracts of *T. divaricata* (0.1 mg/ml) inhibit more than 90% of AChE activity in their *in vitro* study. However, neither its effects on the AChE inhibition nor its neuronal activity enhancement in cerebral cortex, a brain region critical for learning and memory, have ever been investigated in an *in vivo* model. Therefore, in the present study, we tested the hypothesis that the acute administration of *T. divaricata* extract (TDE) can 1) enhance neuronal activity in cerebral cortex and 2) inhibit the activity of circulating and cortical acetylcholinesterase (AChE) as well as butyrylcholinesterase (BuChE). To the best of our knowledge, this is the first study investigating these effects of TDE in rats.

วิธีการทดลอง

Plant materials

T. divaricata was collected from Phitsanulok, Thailand. The voucher specimen (collection no. Changwijit 0020) was deposited at the PBM herbarium, Faculty of Pharmaceutical Sciences, Mahidol University, Thailand.

Extract of *T. divaricata*

Roots of *T. divaricata* were separated from the whole plants and dried at 55 ºC. The dried materials were ground, macerated with 95% ethanol twice (for 3 and 7 days) and dried by evaporating the ethanol extracts under a reduced pressure. To confirm the quality of TDE in each experiment, each lot of TDE was microplate assayed for AChE and BuChE activity *in vitro* prior to its use in rats.

In vitro *analysis for AChE and BuChE activity*

The assay for measuring ChE activity was modified from the assay described by Ellman et al. (1961) and Ingkaninan et al. (2003). Briefly, 125 µl of 3 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 25 µL of 15 mM acetylthiocoline iodide (ATCI) for AChE activity or butyrylthiocoline idoide (BTCI) for BuChE activity, 50 µl of buffer, and 25 µl of

sample dissolved in buffer containing not more than 10% methanol were added to microplate wells, followed by 25 µl of 0.28 U/ml AChE. The microplate was then read at 405 nm every 5 sec for 2 min by a CERES UV 900C microplate reader (Bio-Tek instrument, USA). The velocities of the reactions were measured. Enzyme activity was calculated as a percentage of the velocities compared to that of the blank sample. Inhibitory activity was calculated by subtracting the percentage of enzyme activity from one hundred percent. Every experiment was done in triplicate. Stock solutions of samples in Trisma hydrochloride (Tris-HCl) buffer containing not more than 10% methanol was diluted serially with Tris-HCl buffer to obtain eight or nine different concentrations. The IC_{50} value, the inhibitory concentration that caused 50% inhibitory activity, was analyzed using the software package Prism (Graph Pad Inc, San Diego, USA).

***In vivo* study protocol**

A total of sixty male Wistar rats, weighing 100-150 g, were purchased from the National Animal Center, Salaya campus, Mahidol University, Bangkok, Thailand. All rats were housed, two or three per cage, with free access to food and water for at least 1 week prior to the study. The research protocol adhered to the “Guide for the Care and Use of Animals in compliance with National Institute of Health guideline for the care and treatment of animal” and followed appropriate Faculty of Medicine, Chiang Mai University Standard Operating Procedure for animal identification (approved protocol number 07/2547), housing and diet before and during the experiments.

For the study of neuronal activity in the cerebral cortex, the Fos immunohistochemistry technique was used. In this study, rats were divided into 4 groups (n=6 per group). Each group was injected intraperitoneally with one of the following concentrations of TDE dissolved in ethanol (EtOH); 250 mg/kg (group 1), 500 mg/kg (group 2), or 1 g/kg (group 3). Control animals (group 4) were intraperitoneally injected with EtOH alone. The minimum concentration of TDE used in this study was 250 mg/kg since this concentration was half of the lowest concentration (500 mg/kg) reported to affect parasympathetic activity, such as vasodilatation or mild respiratory depression in animal models (Taesotikul et al., 1989). Since Fos protein exhibits maximal expression after two hours of stimulation, each rat was allowed to survive for two hours after TDE injection before being sacrified for Fos immunohistochemistry study (Chattipakorn et al., 1999).

For the study of ChE activity, rats were divided into 4 groups (n=9 per group). Each group was injected intraperitoneally with 250 mg/kg (group 1), 500 mg/kg (group 2) or 1 g/kg (group 3) of TDE dissolved in EtOH. Control animals (group 4) were intraperitoneally injected with EtOH alone. Each animal was allowed to survive for two hours after injection of TDE before being sacrified. In all rats, blood and cerebral cortex were collected for ChE activity determinations.

Fos immunohistochemistry

To address the possibility that TDE enhanced neuronal activity, we determined the expression of *c-fos proteins* in cortical neurons following acute TDE administration. *C-fos* is a cellular marker in which the pattern of its staining can provide clues about the involvement of specific neuronal populations as being involved in learning-associated signal processing or as having been activated by external stimuli (Aggleton and Pearce, 2001; Bozon et al., 2002; Marcus et al., 1998). Therefore, if neurons are activated via TDE, Fos-positive neurons in brain tissues, particularly cerebral cortex, following TDE administration should be observed. In this study, each animal was deeply anesthetized two hours after TDE injections with pentobarbital (80 mg/kg, intraperitoneally) and perfused intracardially with PBS, followed by 4% paraformaldehyde. The whole brain was removed, postfixed and placed in 30% sucrose overnight and then frozen and cut into 40-µm-thick, transverse frozen sections. Every section was processed for Fos, using a rabbit polyclonal antibody (Santa Cruz, 1: 1,000 dilution). A black reaction product was produced with a standard ABC reaction (Vectastatin Elite kit; Vector Labs) with nickel intensification using Vectastain, and sections were mounted on slides. Processing for Fos was similar to that described previously (Chattipakorn et al., 1999).

ChE activity determinations in blood and cortical samples

Determination of ChE activity was based on the colorimetric method originally described by Ellman et al (1961), adapted for determining the enzyme activity in rat blood and cortical homogenates.

The fresh cortical tissues were weighed and then homogenized in 10 parts of 0.1 M phosphate buffer pH 7.4, which contains 1% Triton-X 100. Following centrifugation at 15,000 rpm for 15 minutes at 4^{o}C, the clear supernatants were removed and served as the enzyme source. AChE activity was determined in 50 µl aliquots of RBC or the cerebral homogenates (run as duplicates). The reaction was started by adding 1) 0.5 mM acetylthiocoline-iodide (ATCI), the commonly used substrate for *in vitro* AChE

determinations (Ellman et al., 1961) or 2) 0.5 mM butyrylthiocoline iodide (BTCI), the commonly used substrate for *in vitro* BuChE determinations, and 0.25 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DNTB), both dissolved in phosphate buffer pH7.4 (0.1M). The plate was then immediately placed into the automatic microplate reader and the yellow reaction product was quantified at 22^{o}C using a wavelength of 405 nm. The reaction was monitored over a period of 10 minutes with readouts taken every 10 seconds. The reaction was then processed by a program controlled by the plate reader. Data were stored on a computer. Quantification of the enzymatic activity was based on a change in optical density in the linear range over time, using the molar extinction coefficient of the reaction products. The spectophotometric absorption was quantitatively measured and expressed as nmol acetylcholine hydrolysed/min/ml RBC or mg cortical tissue and nmol butyrylcholine hydrolysed/min/ml plasma or mg cortical tissue. All chemicals used in this study were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Data analysis

For Fos immunohistochemistry, changes in the number of *c-fos*-positive cells in the cortical regions between different treatment groups were counted using a double blind technique (Chattipakorn et al., 1999). Data from Fos immunohistochemistry and ChE activity were expressed as means$\pm$SE. Statistical analyses were carried out using non-parametric analysis (Kruskal Wallis test) and the Mann Whitney-U test for post-hoc testing to calculate significance for both TDE-treated and control rats. Significance was set at $P < 0.05$.

ผลการทดลอง

TDE can inhibit cholinesterase activity in the microplate assays.

The ethanolic extracts of TDE were tested for AChE and BuChE activity using Ellman's colorimetric method in 96-welled microplates. The concentration of TDE that inhibited 50% of AChE and BuChE activity (IC_{50}) was 2.56 $\pm$ 0.37 and 76.95 $\pm$ 0.11 mg/l, respectively. The AChE inhibitory effect of TDE from microplate assay was less potent than that of galantamine (Figure 1.1). These findings confirm those of a previous report using methanol extracted TDE (Ingkaninan et al., 2003) and suggest that TDE is a ChE inhibitor, but it inhibits AChE more than BuChE in this *in vitro* study.

TDE increases neuronal activity in cerebral cortex in a dose-independent manner.

In this study, we demonstrated that TDE can induce Fos-like immunoreactivity (FLI) in the nuclei of the cerebral cortex at two hours after TDE administration. No FLI-positive neurons were found in the cortical sections, in which the primary antibody was omitted (Figure 1.2a). The FLI-positive neurons were scattered all over the cerebral cortex in all TDE-treated groups (Figure 1.2b-Figure 1.2e). The number of FLI-positive neurons per animal in cerebral cortex were 101±14, 124±19 and 108±22 in the groups administered 250, 500 and 1000 mg/kg TDE, respectively. The numbers of cortical FLI-positive neurons in all three TDE-treated groups were greater ($P<0.05$) than those in the control group (49±9, Figure 1.3). However, FLI-positive neurons in cerebral cortex among three doses of TDE-treated groups were not significantly different (P = 0.1). These results suggest that TDE can enhance neuronal activity in the cerebral cortex in a dose-independent manner for the concentrations used in this study and possibly act via increasing acetylcholine.

TDE inhibits cortical AChE activity but does not inhibit cortical BuChE activity and circulating ChE activity.

The percentage inhibition of AChE activity compared to control in cerebral cortex was 17.4±6.3%, 22.7±6.9% and 16.6±5.0% for 250, 500, and 1000 mg/kg TDE administration, respectively. These AChE activities from TDE groups were significantly different from those in the controls ($P<0.05$, Figure 1.4). No difference was found among these TDE-treated groups ($P=0.39$, Figure 1.4). In contrast to AChE activity, % BuChE inhibitory effects in the cerebral cortex of all three TDE-treated groups were 12.3±5.3%, 15.2±5.2% and 8.6±4.4% for 250, 500, and 1000 mg/kg TDE, respectively (Figure 1.4). BuChE activity in cerebral cortex from all three TDE treated group were not significantly different from those in the controls ($P=0.1$). In the positive control study, we found that the acute single administration of 10 mg/kg galantamine intraperitoneally (n=4) can inhibit activity of cortical AChE (28±6%) and BuChE (0.6±4%) compared to what was seen in the controls (0 mg/kg galantamine; n=4). We used 10 mg/kg galantamine as a positive control for TDE administration because this dose had been previously reported to have AChE inhibitory effects in mouse forebrain (Bores et al., 1996d). The percentage of AChE and BuChE inhibitiory activities in cortical tissues at two hours after galantamine injection was similar to those of TDE administration reported in the present study.

In contrast to the findings in cortical tissue, TDE had no effect on ChE activity in the circulation, either in erythrocyte AChE or plasma BuChE activities at two-hours after TDE administration (Figure 1.5). The percentage inhibition of circulating AChE and BuChE activities two hours after 10-mg/kg-galantamine administration was 2.6±1% and 5±1%, respectively. The circulating ChE activity after galantamine administration was not significantly different from that in the controls (*P=0.063*). Our results are consistent with a previous report which demonstrated that the AChE inhibitory effects of galantamine in circulation were insignificant two hours after administration (Bores et al., 1996c). These findings indicate that TDE may be a short-acting and reversible agent in inhibiting ChE activity in the circulation, similar to galantamine.

อภิปรายและวิเคราะห์ผล

The major finding of this study is that TDE can inhibit AChE activity in cerebral cortex in rats, as well as in an *in vitro* study. Our study also demonstrated, for the first time, that TDE can enhance neuronal activity and has cortical AChE inhibitory effects in an animal model.

Effect of TDE on the cerebral cortex

Our results demonstrated enhanced cortical neuronal activity at two hours following the administration of TDE, as indicated by an increase in Fos-positive neurons. In this study, cerebral cortex was chosen to determine neuronal activity and ChE activity, since it plays an important role in learning and in memory and is generally the main representative of the central cholinergic innervation (Warburton et al., 2003). The analysis of immediate early gene (IEG), particularly Fos, induction is a useful tool for investigating activated neuronal populations. The induction of IEGs, i.e. Fos, is rapid, transient and protein synthesis independent (Curran and Morgan, 1995; Hughes and Dragunow, 1995; Morgan and Curran, 1990). The protein products of IEGs are generated in the cell membrane, in cytosol and, importantly, in the nucleus; thus these locations are ideally suited for the regulation of gene expression. In normal physiological states, the basal level of IEG expression in the brain is low. However, different stimuli can induce IEG in neurons of CNS structures known to be involved in the processing of these stimuli. A previous study also demonstrated that the administration of AChE-Is can lead to endogenous acetylcholine (ACh)-induced Fos expression in the supraoptic nucleus of the rat hypothalamus (Shen and Sun, 1995). Therefore, the enhancement of Fos expression in cortical neurons following TDE administration as shown in this study

suggests that TDE may cause an increase of endogenous ACh in cerebral cortex, resulting in an increase in the cortical activity similar to that observed in other AChE-Is.

In the present study, we also demonstrated that TDE could inhibit neuronal AChE activity in a dose-independent manner at two hours after administration, but had no effect on cortical BuChE at that time point. These results are consistent with our *in vitro* study, in which TDE was shown to inhibit AChE activity more than BuChE activity. These findings suggest that TDE may be a selective AChE-I similar to donepezil and galantamine (Ballard, 2002). In contrast to our *in vitro* study, in which the AChE inhibitory effect of TDE was ten times less than that of galantamine, it is important to note that the cortical AChE inhibitory effect in the *in vivo* study two hours after TDE administration was the same as that of galantamine. The percentage inhibition of cortical AChE activity following galantamine administration in our study is consistent with reports from previous animal studies (Geerts et al., 2005; Bores et al., 1996). Therefore, the differences between *in vitro* and *in vivo* results may possibly be due to the differences in bioavailability of active compounds in TDE and in galantamine. The metabolic rate of galantamine may possibly be greater than that of TDE in animal models. Previous studies demonstrated that levels of galantamine in the brain after 10 mg/kg subcutaneous injection increased transiently with a maximum between 15 and 30 min and clearance out of the brain was rapid (Bickel et al., 1991; Bores et al., 1996). Both preclinical and clinical evidence suggest that there is no accumulation of galantamine over time in rats (Geerts et al., 2005; Mannens et al., 2002). In addition, the whole-brain AChE inhibition of galantamine in rats decreased over time and galantamine's activity is essentially terminated three hours after administration (Geerts et al., 2005). Therefore, future investigations on TDE bioavailability are necessary.

The augmentation of neuronal activity following TDE administration could result from at least two possible mechanisms. First, TDE acts as an AChE-I in animals as it does in *in vitro*. AChE is an esterase critical in the metabolism of ACh at central and peripheral synapses (Koelle, 1963). If AChE activity is inhibited, ACh levels should be increased. ACh is plentiful and more widely distributed in the brain than in any other neurotransmitters. The cholinergic system is capable of keeping the neocortex operative (Giacobini, 2003). Therefore, high ACh levels in the cerebral cortex could lead to an increase in cortical neuronal activity. The evidence that TDE acts as a cortical AChE-I in an animal model as demonstrated in the present study supports this hypothesis. Another possible mechanism of TDE in the enhancement of cortical neuronal activity

could be that TDE may serve as an allosteric potentiating ligand (APL) binding at the nicotinic ACh receptor (nAChR), and can directly potentiate neuronal activity. It has been shown that galantamine is an APL at the nAChR (Woodruff-Pak et al., 2001). Since TDE can inhibit AChE activity and can show characteristics similar to galantamine, it is, therefore, possible that TDE could enhance cortical neuronal activity via this mechanism. However, further investigations are needed to justify this hypothesis.

There are several possible active compounds in TDE that could play a role as AChE-Is. The *T. divaricata* specimens used in the present study were in the form of crude extract. This crude extract consisted of at least forty-four alkaloids (Atta-Ur-Rahman et al., 1985; Henriques et al., 1996; Pawelka and Stockight, 1983),and non-alkaloid constituents; such as triterpenoids (Rastogi et al., 1980; Sharma and Cordell, 1988; Van Der Heijden, 1989), steroids (Dagnino et al., 1991; Sharma and Cordell, 1988), flavonoids (Daniel and Sabnis, 1978), phenyl propanoids (Dagnino et al., 1991; Daniel and Sabnis, 1978) and phenolic acids (Henriques et al., 1996). Previous studies have shown that several alkaloids in TDE, such as Coronaridine (Andrade et al., 2005), Voacangine (Andrade et al., 2005) and Isovoacristine (Raymond-Hamet, 1962), have anti-AChE activity *in vitro*. Therefore, the inhibitory effects of AChE activity in our animal model could be due to the effect of mixed alkaloids in TDE. The inhibitory AChE effects of each pure alkaloid in TDE also need to be determined in future studies.

Effect of TDE in the circulating ChE activity

Our study demonstrated that the mean percentage of ChE inhibition of TDE in circulation was insignificant two hours after TDE administration. These results were similar to those previously observed after galantamine administration. Since previous studies have shown that the inhibition of enzyme activity following galantamine administration reduces over time, it is possible that the inhibitory effects of TDE may also be time-dependent (Geerts et al., 2005; Sweeney et al., 1989; Van Beijsterveldt et al., 2004). If rapid clearance of TDE is the cause of the short term-inhibition of circulating AChE and BuChE activity following a single-dose TDE administration, any change of circulating ChE activity of TDE may not be observed, as shown in this study. Future studies are needed to investigate the bioavailability of TDE.

In summary, preclinical analyses using *in vivo* enzymatic techniques in the present study demonstrate that TDE is a selective AChE inhibitor and can enhance neuronal activity, suggesting that it may be a candidate for the treatment of AD. Future investigations on the behavioral studies, therapeutic indices, pharmacokinetics and

complete toxicological evaluation of TDE are necessary to evaluate its definitive therapeutic benefits.

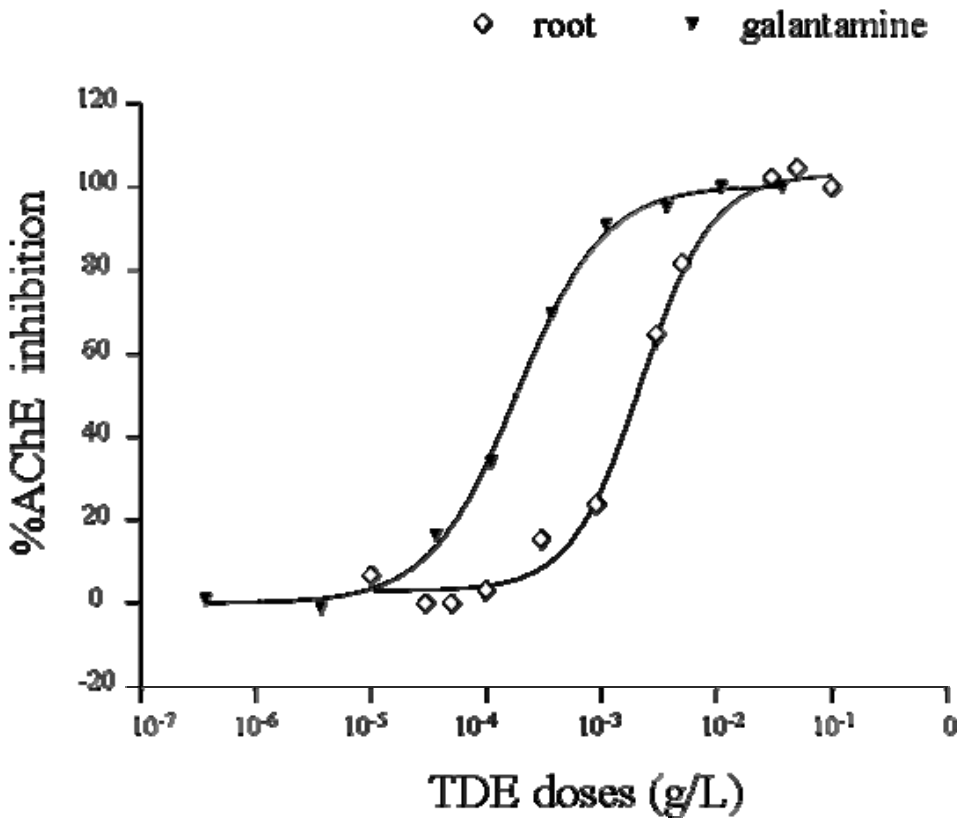

Figure 1.1: The dose response curves of % AChE inhibition from *Tabernaemontana Divaricata* extract (◊) in comparison with galantamine (▼). Values are means of one typical experiment performed in triplicate.

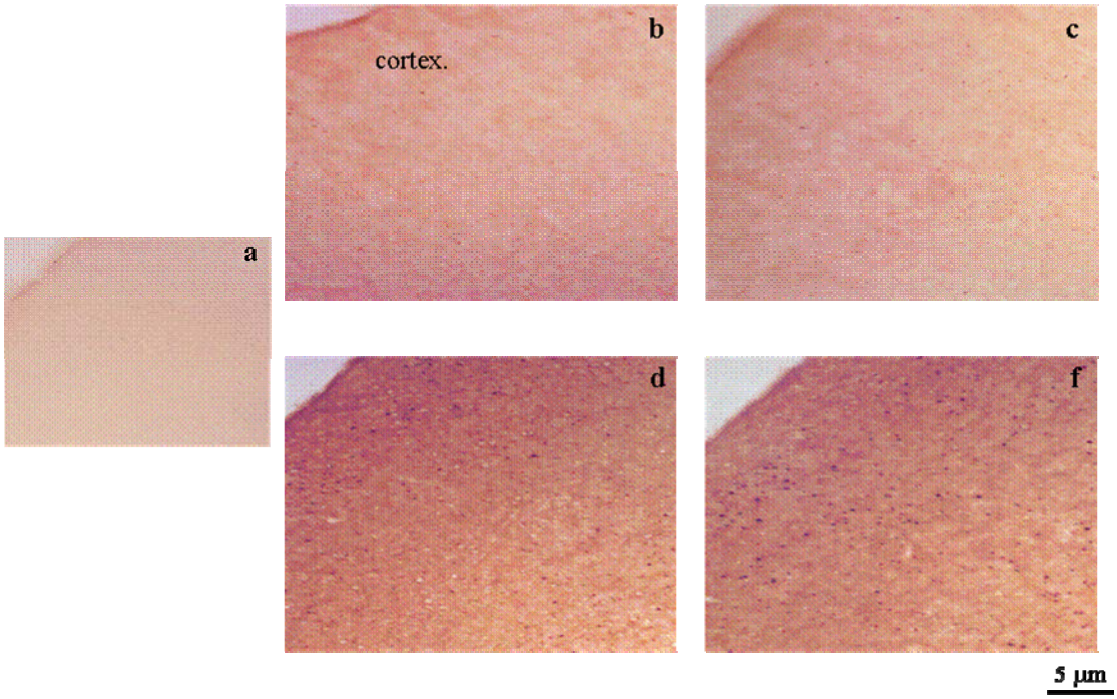

Figure 1.2: Representations of Fos-positive neurons in cerebral cortex 2 hours after intraperitoneal injection of various doses of TDE. (a) Negative control represents the cortical tissue from the 1000 mg/kg TDE-treated group with the primary Fos antibody omission. b-e represents the cortical tissue 2 hours after TDE injection (0 mg/kg (b), 250 mg/kg (c), 500 mg/kg (d) and 1000 mg/kg (e)).

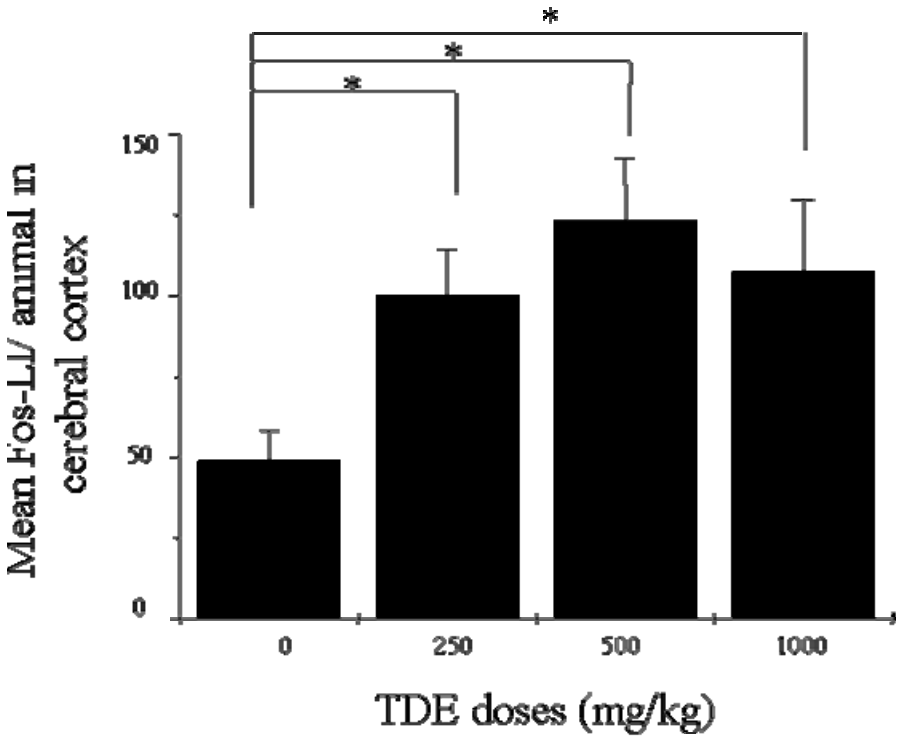

Figure 1.3: Mean Fos-positive neurons per animal in cerebral cortex 2 hours after intraperitoneal injection of various doses of TDE (n=6 per group). *: *p<0.05* compared to control group (0 mg/kg of TDE).

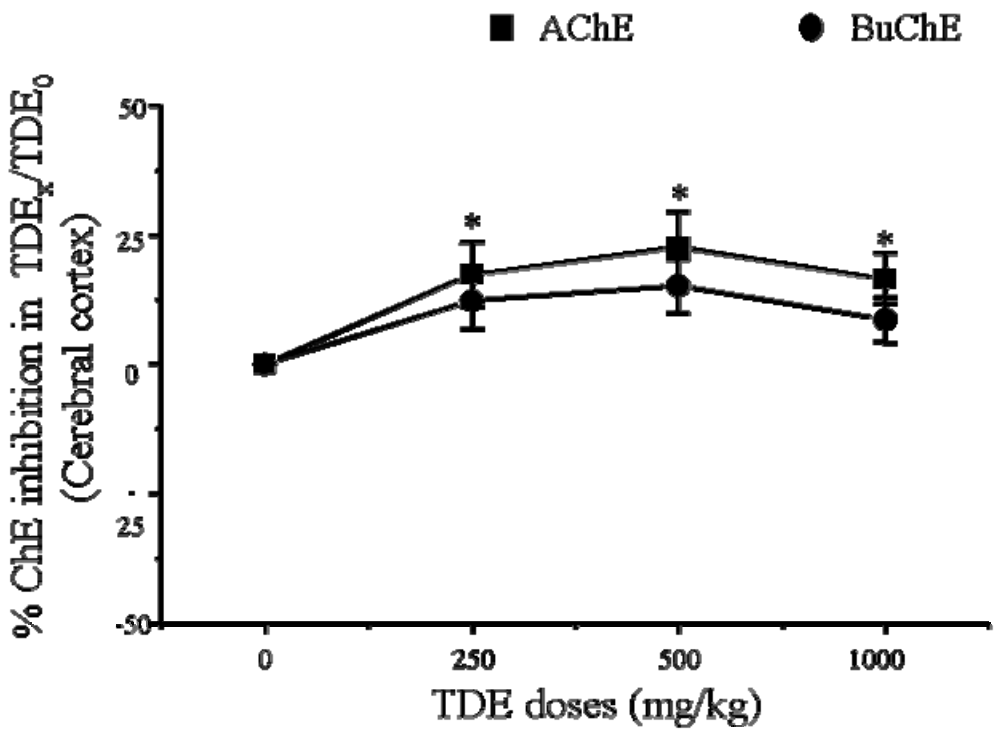

Figure 1.4: Normalized % cortical ChE inhibition levels in rats (TDE_x/TDE_0) after intraperitoneal injection of various doses of TDE (TDE_x). TDE_0 represents the control group (0 mg/kg of TDE). Each data point represents mean±SE. AChE = acetylcholinesterase, BuChE = butyrylcholinesterase *: $p<0.05$ compared to control group (0 mg/kg of TDE = TDE_0).

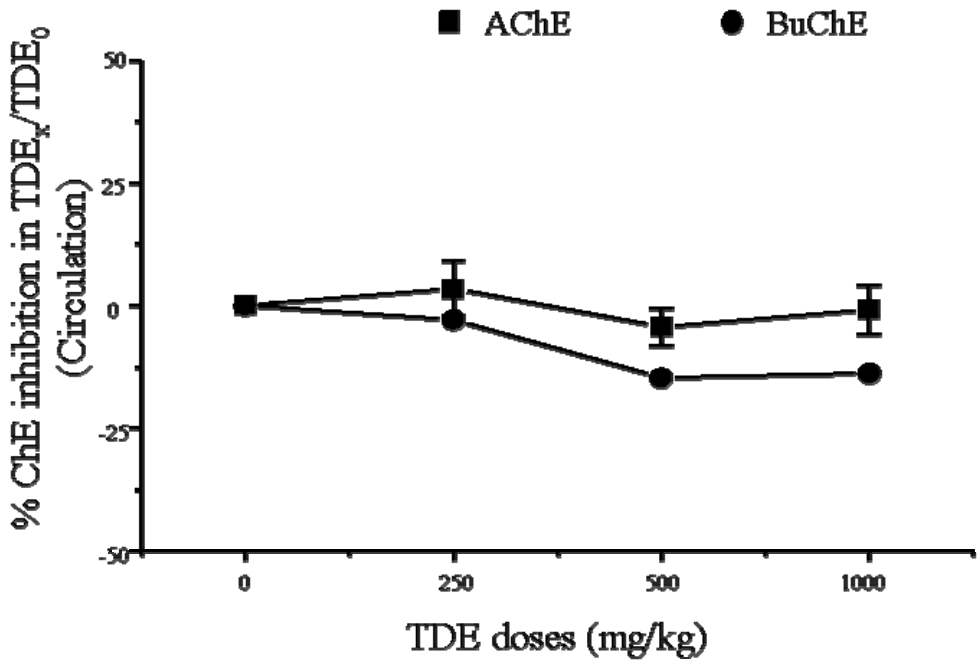

Figure 1.5: Normalized % circulating ChE inhibition levels in rats (TDE_x/TDE_0) after intraperitoneal injection of various doses of TDE (TDE_x). TDE_0 represents the control group (0 mg/kg of TDE). Each data point represents mean±SE. AChE= acetylcholinesterase, BuChE = butyrylcholinesterase

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บทที่ 2: THE REVERSIBLE ACETYLCHOLINESTERASE INHIBITOR EFFECT OF *TABERNAEMONTANA DIVARICATA* EXTRACT ON SYNAPTIC TRANSMISSION IN RAT CA1 HIPPOCAMPUS

บทนำ

Acetylcholine (ACh), acting through neuronal muscarinic acetylcholine receptors (mAChRs) and nicotinic acetylcholine receptors (nAChRs), is an important modulator of electrical activity in the central nervous system. Acetylcholine is involved in a variety of physiological processes and in synaptic plasticity, including cognition and development (Qian and Saggau, 1997; Hasselmo and Fehlau, 2001; Levin, 2002; Volpicelli and Levey, 2004). Several studies show that ACh modulates excitatory transmission throughout the brain. For example, the stimulation of cholinergic inputs to the hippocampus decreases the size of synaptic responses in glutamatergic projections in different regions of the hippocampus (Yamamoto and Kawai, 1967; Konopacki et al., 1987; Kahle and Cotman, 1989; Foster and Deadwyler, 1992; Hasselmo et al., 1995; Qian and Saggau, 1997; Hasselmo and Fehlau, 2001; Gipson and Yeckel, 2007). Modulation of the synaptic strength of excitatory glutamate synapses in the hippocampus is believed to be involved in memory processing (Bliss and Collingridge, 1993). Colgin and colleagues also showed that the acetylcholinesterase inhibitor (AChE-I), physostigmine, enhanced cholinergic transmission and subsequently depressed glutamate release in hippocampal pathways (Colgin et al., 2003).

The loss of cholinergic function has been implicated in Alzheimer's disease (AD), the leading causes of dementia (Terry and Buccafusco, 2003). Symptomatic pharmacological treatment of AD is mainly based on the use of AChE-Is, such as donezepil, rivastigmine, and galantamine, which have beneficial effects on cognitive, functional, and behavioral symptoms of AD as well as undesired side effects (Giacobini, 2004). The need for novel treatments and the fact that the role of cholinesterase inhibitors in AD are still not completely unveiled, have led to the investigation of new natural AChE-Is. *Tabernaemontana divaricata (L.) R. Br. Ex Roem. & Schult* (*T. divaricata*) is a common garden plant in tropical countries. It has been reported as a rich source of alkaloids with various pharmacological properties (Van Beek et al., 1984). Ingkaninan and colleagues have shown *in vitro* that ethanol extracts from *T. divaricata* root (TDE) at a concentration of 0.1 mg/ml inhibit more than 90 % of acetylcholinesterase (AChE) activity (Ingkaninan et al., 2003). In addition, we have recently shown that the extract from *T. divaricata* acts as a novel reversible neuronal

AChE inhibitor in an animal model (Chattipakorn et al., 2007). However, the study of cholinergic effects using TDE, as an AChE-I, on the hippocampal circuit has not been investigated. This study tested the effects of the AChE inhibitor, TDE, on dendritic field excitatory postsynaptic potentials (fEPSPs) in CA1 stratum radiatum from hippocampal slices of normal rats. Our findings indicate that TDE-enhanced cholinergic transmission in hippocampal circuits affects synaptic glutamatergic transmission by depressing neurotransmitter release in a similar manner to the effect of exogenous galantamine and acetylcholine application. These data suggest further that the effect of TDE is mediated by muscarinic acetylcholine receptors (mAChRs) rather than by nicotinic acetylcholine receptors (nAChRs).

วิธีการทดลอง

Plant materials and Extract of T. divaricata

T. divaricata (collection No. Changwijit 0020 at the PBM herbarium, Faculty of Pharmaceutical Sciences, Mahidol University, Thailand) was collected from Phitsanulok, Thailand. Roots of *T. divaricata* were extracted as described in our previous study (Chattipakorn et al., 2007). To validate the quality of TDE in each experiment, each lot of TDE was analyzed for the inhibitory effects of AChE activity *in vitro* and *in vivo* before being used in this study.

Hippocampal slice preparation

All experiments described in this manuscript were conducted with an approved protocol from the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. Hippocampal slices (400 μm) were prepared from 4- to 5-week-old male Wistar rats (weight 100-150 g, n=23) using standard methods. Rats were anesthetized with halothane, decapitated, and the brain removed and placed in ice-cold “high sucrose” aCSF containing (mM): NaCl 85; KCl 2.5; $MgSO_4$ 4; $CaCl_2$ 0.5; NaH_2PO_4 1.25; $NaHCO_3$ 25; glucose 25; sucrose 75; kynurenic acid 2; ascorbate 0.5, saturated with 95%O_2/5%CO_2 (pH 7.4). The low Na^+ and Ca^{2+} and high sucrose content of this solution enhanced neuronal survival during the slicing procedure. Coronal slices from the dorsal hippocampus were cut using a vibratome (The Vibratome Company, St. Louis, MO, USA). Following a 30-min post-slice incubation in high sucrose aCSF, slices were transferred to a standard aCSF solution containing (mM): NaCl 119; KCl 2.5; $CaCl_2$ 2.5; $MgSO_4$ 1.3; NaH_2PO_4 1; $NaHCO_3$ 26;

and glucose 10, saturated with 95% O_2/5%CO_2 (pH 7.4) for an additional 30 minutes. For recordings, the slices were transferred to a submersion recording chamber and continuously perfused at 3-4 ml/min with standard aCSF (described above), warmed to 25-28°C.

Stimulation and recording

CA1 extracellular dendritic field excitatory postsynaptic potentials (fEPSPs) were recorded (Axopatch 200B, Axon Instruments, CA, USA) using standard methods, as described in a previous study (McMahon and Kauer, 1997). A stainless steel bipolar stimulating electrode (FHC, Bowdoinham, ME, USA) was placed in stratum radiatum to stimulate the Schaffer collaterals, and a glass microelectrode filled with 2M of NaCl was placed in CA1 stratum radiatum to record fEPSPs. The stimulus frequency was 0.1 Hz (100 μsec duration). The stimulus intensity was adjusted to yield a field EPSP of 0.8-1.0mV in amplitude and produce ~50% of maximal fEPSP responses. The delivery of two stimuli in rapid succession (50-msec interstimulus interval) elicited paired-pulse facilitation (PPF).

Drug application

Various compounds were used in this experiment, including TDE (dissolved in ethanol, concentration listed in μg/ml), atropine (dissolved in ddH_2O, concentration listed in μM), pancuronium bromide (dissolved in ddH_2O, concentration listed in μM), ACh and galantamine (dissolved in ddH_2O, concentration listed in mM and μM, respectively). Appropriate concentrations of specific test substances in solution were determined experimentally. In the baseline and wash condition, hippocampal slices were perfused with standard aCSF. However, the same amount of ethanol was added to the standard aCSF in the baseline and wash condition for the TDE experiment as was used for dissolving TDE in the experiment. All chemical substances were ordered from Sigma (St. Louis, MO, USA). All substances were prepared on the day of the experiment and applied to the slices in a bath chamber via gravity perfusion.

Data analysis

Data were filtered at 3 kHz, digitized at 10 kHz, and stored on a computer using pclamp 9.2 software (Axon Instrument, CA, USA). The initial slope of the fEPSP was measured and plotted vs. time, with each point representing a single raw data point. Statistical

significance between the groups was determined with the Student's t test. Significance was determined at $p < 0.05$. Data were presented as mean ± SEM. Only experiments with less than a 10% change in the original baseline were included in the analysis.

ผลการทดลอง

The effect of *T. divaricata* extract (TDE) on extracellular dendritic field excitatory postsynaptic potentials (fEPSPs) recorded in the stratum radiatum of the CA1 hippocampus, in response to stimulation of the Schaffer collaterals, is demonstrated in Figure 2.1. Paired-pulse facilitation (PPF), in response to paired stimulation pulses, was used to clarify the Schaffer collateral pathways (Figure 2.1A). TDE (60 μg/ml) reduced the size of the responses beginning 2-3 minutes after the start of infusion, with maximum effects appearing over the following 5-7 minutes. In the apparatus used in these experiments, compounds added to the infusion line required approximately 1 minute to reach the slices. The fEPSP depression was prominent and did not appear to be accompanied by distortion of waveform (Figure 2.1A, inset). The fEPSP responses returned to the same level as the responses in the baseline after 15 minutes of washout (Figure 2.1A). The mean depression of fEPSPs following the application of 60 μg/ml of TDE was 47 $\pm$ 4% (n=7, Figure 2.1B). We used 60 μg/ml of TDE in this study because this concentration of TDE showed the maximal effect on the depression of fEPSPs. TDE with concentrations higher than 10 μg/ml transiently reduced synaptic responses. The range of fEPSP depression in TDE concentrations of 10-100 μg/ml was 46-75% (n = 5 per dose).

Figure 2.2 illustrates the results obtained in CA1 hippocampal slices and shows that the effects of 60 μg/ml of TDE were completely blocked by 10 μM of atropine, a muscarinic AChR antagonist (Figure 2.2A and 2.2B, n=7). It appeared that the effect of TDE was mediated by muscarinic ACh receptors. We used a high concentration of atropine (10 μM) in this experiment because atropine (10 μM) can completely block the effect of TDE on the reduction of synaptic response in all hippocampal slices (n=7).

To investigate whether the nicotinic acetylcholine receptors (nAChRs) were also involved in the synaptic modulation of TDE, we performed fEPSP experiments using pancuronium bromide, a non-selective nAChR antagonist, with and without TDE application. In contrast to the effect of atropine, the TDE-induced reduction in Schaffer collateral fEPSPs persisted despite pretreatment of the slices with pancuronium bromide (100 μM). The mean depression of fEPSPs following the application of 60 μg/ml of TDE

with and without 100 μM pancuronium was not significantly different. ($p < 0.05$, n=6). We used a high concentration of pancuronium (100 μM) to completely block all subtypes of nAChR in hippocampal regions. This finding revealed that the effect of TDE in synaptic modulation should not be mediated by nAChRs.

In comparing the fEPSP suppression of 60 μg/ml of TDE and of 1 μM galantamine; a reversible AChE inhibitor, we found that the reduction of synaptic responses with TDE and galantamine was similar. We used 1 μM galantamine in this study because it has been demonstrated that this concentration can modulate glutamate synaptic transmission (Santos et al., 2002). Figure 2.3 shows that 1 μM galantamine application leads to transiently depress fEPSPs. The mean fEPSP depression following the application of 1 μM galantamine was 42 $\pm$ 8% (Figure 2.3B, n=4). The effect of galantamine in suppressing fEPSPs was also completely blocked by 10 μM atropine (Figure 2.3A). Our findings suggest that TDE acts as a reversible AChE inhibitor in the suppression of CA1 synaptic responses.

The effect of TDE elicited by recording the depression of fEPSPs in the stratum radiatum of the CA1 hippocampus was similar to that of ACh. Figure 2.4 shows that 1 mM of ACh transiently depressed fEPSPs, similarly to TDE application, and the effect of ACh in suppressing fEPSPs was also completely blocked by 10 μM of atropine (Figure 2.4A). The mean fEPSP depression following the application of 1 mM of ACh was 77 $\pm$ 3 % (n=7, Figure 2.4B). We used 1 mM of ACh in this study because this concentration had the maximal effect on the depression of fEPSPs (seen in Figure 2.4C). The depression of fEPSPs by ACh was dose-dependent and the EC_{50} in depressing fEPSPs occurred at 0.52 $\pm$ 0.7 mM of ACh (n = 5 per each dose, Figure 2.4C).

The depression of synaptic transmission with both TDE and galantamine application was less and slower than that with ACh (Figure 2.5A). This finding suggested that TDE depresses fEPSPs in a similar way to galantamine, an AChE-I, and TDE might not directly suppress fEPSPs in the same fashion as exogenous ACh application. TDE and galantamine, reversible AChE-Is, increased the endogenous ACh level at the synaptic sites. Therefore, the amount of ACh in the synaptic regions from TDE and galantamine application had to be less than the exogenous ACh application. Paired-pulse facilitation (PPF), a simple and sensitive measure of changes in presynaptic neurotransmitter release probability, was used to test the hypothesis that TDE, galantamine and ACh reduce the synaptic responses by presynaptically depressing glutamatergic release. Mean paired-pulse facilitation of the CA1

hippocampus was increased during the application of TDE (60 μg/ml), galantamine (1 μM) and ACh (1mM) (Figure 2.5B). Mean facilitation of the response slope with 60 μg/ml of TDE during infusion was 11 ± 4% greater than the mean facilitation of responses before TDE infusion ($p < 0.05$). Mean facilitation of the response slope with 1 μM galantamine during infusion was 13 ± 1% greater than the mean facilitation of responses before galantamine infusion ($p < 0.05$). The increase of the PPF response slope with 1 mM of ACh during infusion was 35 ± 6% greater than the mean facilitation of responses before ACh infusion ($p < 0.01$). These results suggest that the reduction of synaptic responses following TDE, galantamine and ACh infusion might occur at the presynaptic terminals.

อภิปรายและวิเคราะห์ผล

The major finding of this study was the novel effect of *Tabernaemontana divaricata* extract (TDE) on synaptic transmission in the CA1 hippocampal area. We demonstrated that TDE caused an acute reduction in the CA1 synaptic transmission, possibly via a presynaptic mechanism. The effect of TDE was blocked by atropine, but not by pancuronium. These findings suggest that TDE can modulate synaptic transmission via the muscarinic cholinergic function. The effect of TDE was similar to the effect of galantamine, an AChE inhibitor. These findings also add the novel effect of TDE on synaptic transmission to our previous findings showing that TDE acts as an acetylcholinesterase (AChE) inhibitor in the central nervous system.

TDE transiently depresses synaptic transmission in the CA1 hippocampus via the activation of muscarinic cholinergic receptors.

A massive glutamatergic input from the cortex depends on a collection of afferents releasing a transmitter other than glutamate for synchronizing rhythms in the hippocampus. Acetylcholine is a powerful presynaptic modulator at the glutamatergic synapses (Kahle and Cotman, 1989; Hasselmo, 1999). The cholinergic innervation in the hippocampus is provided from the medial septal nucleus (Dutar et al., 1995). Several studies have shown that the stimulation of cholinergic innervation in different regions of the hippocampus reduces the glutamatergic synaptic transmission (Yamamoto and Kawai, 1967; Konopacki et al., 1987; Kahle and Cotman, 1989; Foster and Deadwyler, 1992; Hasselmo et al., 1995; Qian and Saggau, 1997; Hasselmo and Fehlau, 2001; Gipson and Yeckel, 2007). Much evidence has been provided on the cholinergic interaction with glutamatergic transmission (Aigner, 1995; Hasselmo, 1999;

Terry and Buccafusco, 2003). A recent study showed that the coincident glutamatergic and cholinergic inputs transiently depressed glutamatergic release at the CA1 synapse (Gipson and Yeckel, 2007).

We showed previously that TDE acts as a reversible AChE inhibitor and is capable of increasing neuronal activity in the cerebral cortex, possibly by increasing cholinergic function (Chattipakorn et al., 2007). In this study, we showed that TDE caused a transient depression of synaptic transmission in Schaffer collateral pathways of the CA1 response. The modulation of synaptic response by TDE was similar to the effect of exogenous galantamine and ACh application. These findings, with those from our previous study, suggest that TDE, acting as an AChE inhibitor, possibly elevates the endogenous ACh level at the cholinergic synapses in hippocampal circuits. Therefore, the synaptic modulation of TDE may occur via the cholinergic regulation of neurotransmission. This possibility is supported by the illustration in Figure 2.2, in which the transient depression resulting from TDE application is prevented by blockage of muscarinic acetylcholine receptors. This study showed that the effect of TDE depressing Schaffer collateral responses was blocked by atropine (mAChR blocker), but not by pancuronium (nAChR blocker), indicating that the modulation of synaptic transmission from TDE was mediated by muscarinic ACh receptors. The application of atropine or pancuronium, alone, had no effect on the hippocampal synaptic transmission, in which the application of either drug did not change the slopes of fEPSPs. This finding is consistent with a previous study showing that the blockade of AChRs with pancuronium had no effect on excitatory synaptic transmission in CA1 regions (Chiodini et al., 1998). In addition, TDE effects were similar to the effect of the reversible AChE inhibitor, galantamine, as demonstrated in this study, as well as that of physostigmine, in a previous study (Colgin et al., 2003).

TDE affects synaptic responses via presynaptic mechanism.

Changes in a paired-pulse ratio, due to the application of a drug, typically indicate a presynaptic mechanism of drug action (Creager et al., 1980; Wu and Saggau, 1994). We found that TDE acutely reduces the slope of fEPSPs and the depression is accompanied by an increase in the paired-pulse ratio. Our data suggest that TDE transiently depressed synaptic transmission by decreasing neurotransmitter release at the presynaptic site. In addition, the effect of TDE on the paired-pulse ratio was similar to the effect of exogenous galantamine and ACh application. In agreement with this finding, another study also showed that the AChE inhibitor, physostigmine, depressed

synaptic response in the CA1 hippocampus by reducing glutamatergic release at the presynaptic site (Colgin et al., 2003). Analysis of the paired-pulse results provides some insights into the functional changes arising from cholinergic suppression of glutamatergic synapses.

Our data revealed that the accumulation of ACh at cholinergic synapses, during the application of TDE, can activate muscarinic cholinergic receptors, presumably at the presynaptic sites, and lead to modulation of the synaptic transmission. However, the effect of TDE on synaptic response via postsynaptic mechanisms requires further investigation.

The modulation of the cholinergic septo-hippocampal synapses by the novel AChE inhibitor, TDE, causes a substantial depression of glutamate release from inputs to the CA1 hippocampus. This effect appears to involve actions of muscarinic AChRs in the presynaptic terminals, similar to those of another AChE inhibitor; galantamine. Therefore, TDE could be a new pharmaceutical target for developing new alternative AChE inhibitors for therapeutic purposes in the future.

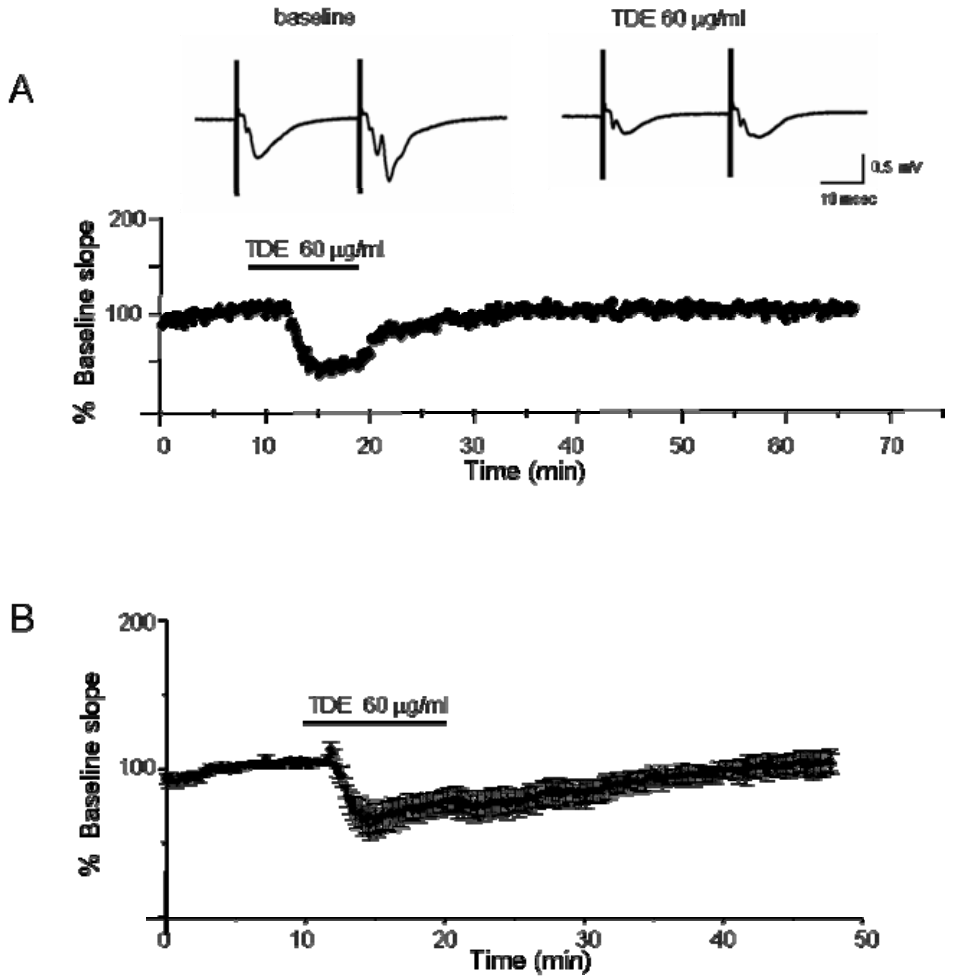

Figure. 2.1: Effect of *Tabernaemontana divaricata* extract (TDE) on the size of CA1 hippocampal responses. Single-pulse stimulation was applied to Schaffer collaterals, and recordings were collected from stratum radiatum. A) A single experiment demonstrated TDE-induced depression of synaptic responses. Traces were obtained from the experiment shown in 1A (inset). Each trace shows the average of 20 consecutive sweeps, recorded 2 minutes before (baseline) and during (TDE 60 µg/ml), TDE application. Scale bar is 0.5 mV and 10 msec. B) Average of 7 TDE application experiments shown as mean ± SEM.

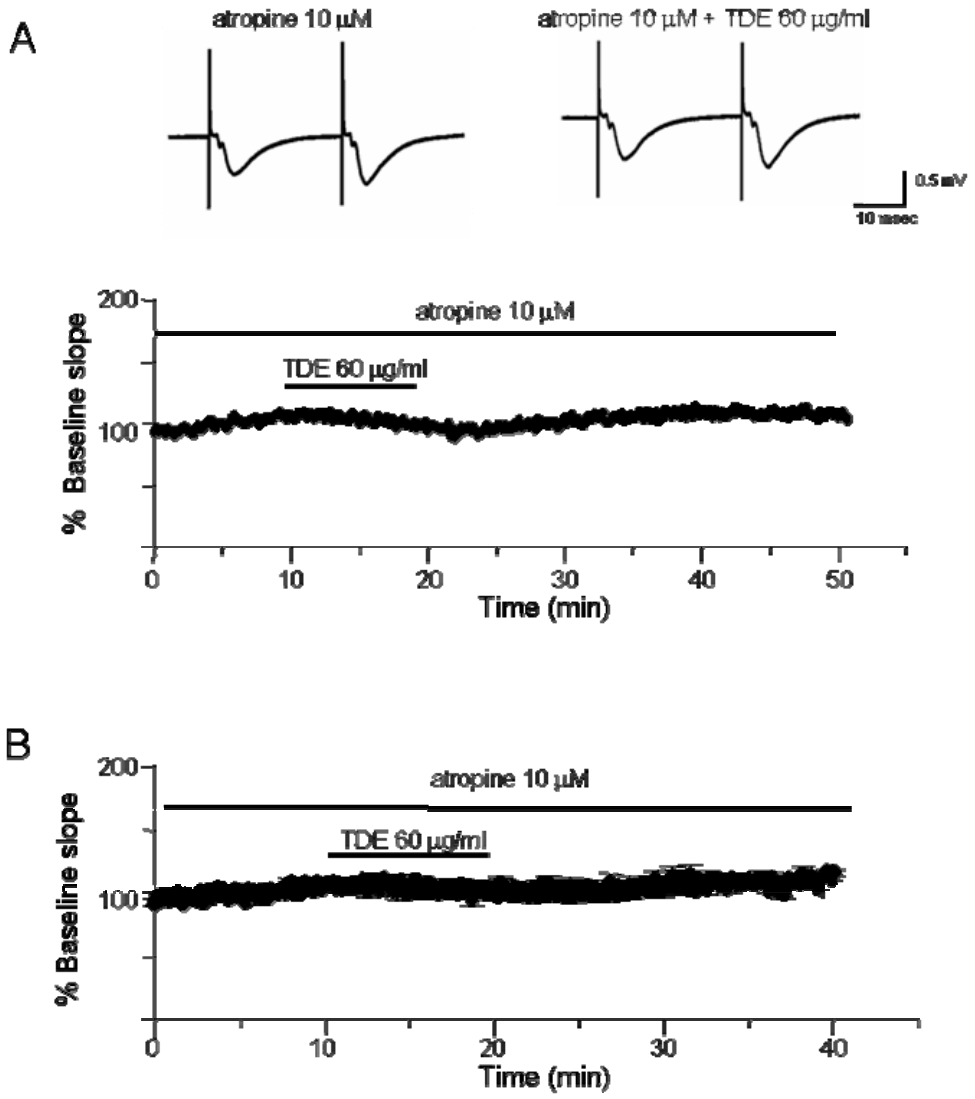

Figure. 2.2: The nonselective muscarinic antagonist, atropine (10 µM), completely blocked the acute synaptic depression caused by TDE. A) A single experiment demonstrated that atropine blocked the TDE-induced synaptic depression. Traces were obtained from the experiment shown in 2A (inset). Each trace shows the average of 20 consecutive sweeps, recorded 2 minutes before the application of TDE (atropine 10 µM) and during the application of TDE (atropine 10 µM + TDE 60 µg/ml application). Scale bar is 0.5 mV and 10 msec. B) Average of 7 TDE application experiments shown as mean ± SEM.

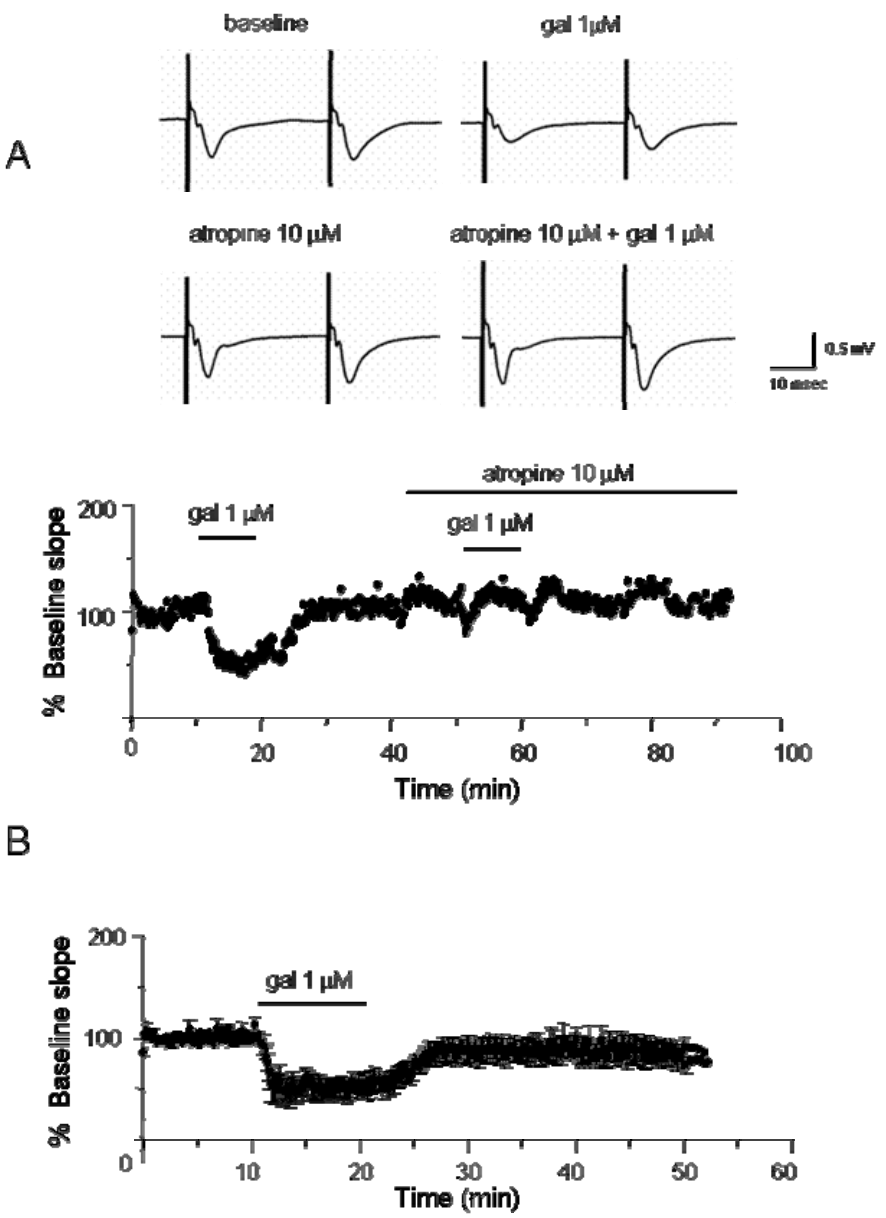

Figure. 2.3: Effect of 1 μM galantamine (gal) on the CA1 hippocampal responses. Single pulse stimulation was applied to Schaffer collaterals, and recordings were collected from stratum radiatum. A) A single experiment demonstrated galantamine-induced depression of synaptic responses, and the depression was blocked by atropine (10 μM). Traces were obtained from the experiment shown in 3A (inset). Each trace shows the average of 20 consecutive sweeps. Scale bar is 0.5 mV and 10 msec. B) Average of 4 galantamine application experiments is shown as mean ± SEM.

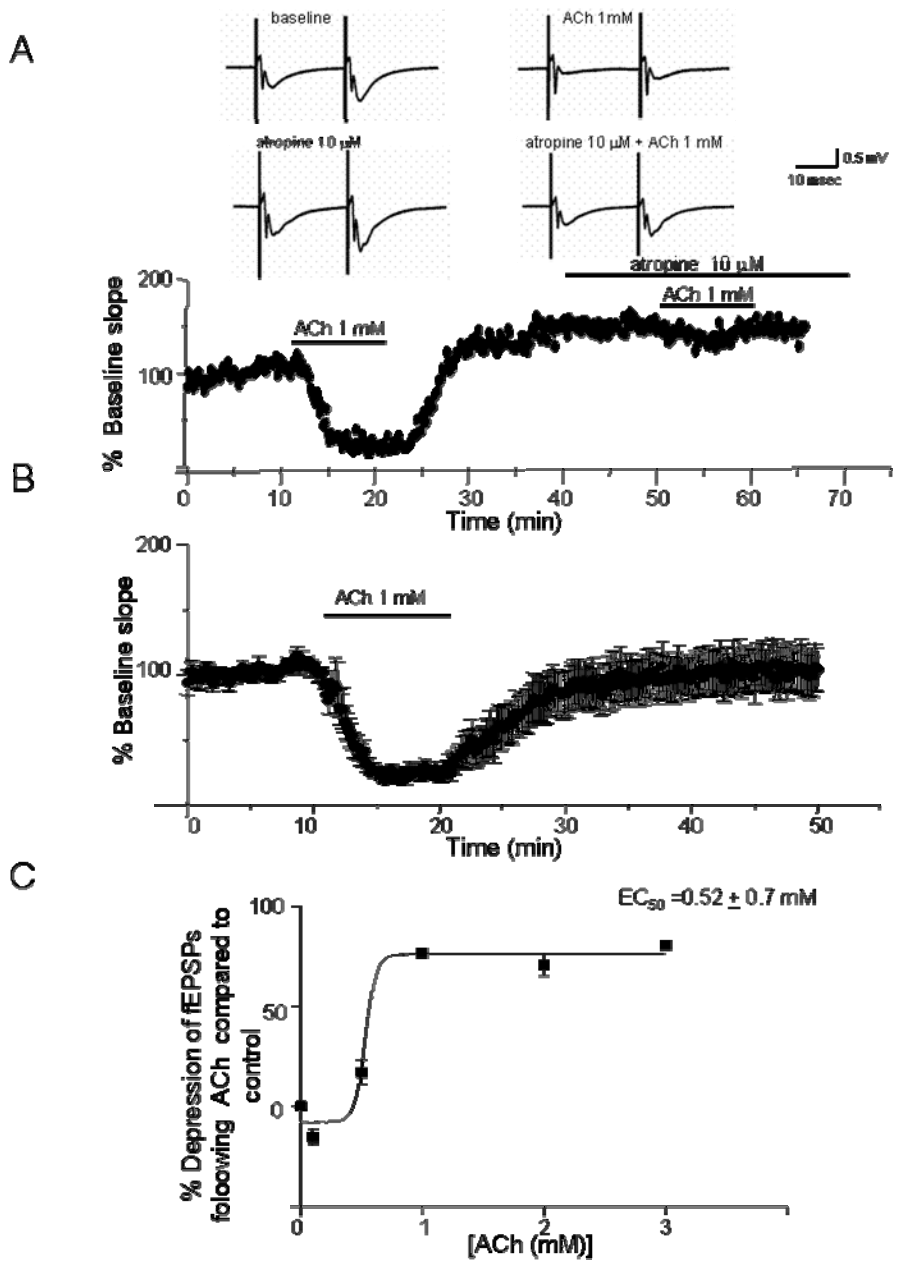

Figure. 2.4: Acetylcholine (ACh) caused a large depression of CA1 hippocampal synaptic response, and that effect was blocked by atropine. A) A single experiment demonstrated ACh-induced depression of synaptic responses. The effect of ACh was blocked by atropine. Traces were obtained from the experiment shown in 4A. (inset). Each trace shows an average of 20 consecutive sweeps. Scale bar is 0.5 mV and 10 msec. B) Average of 7 ACh application experiments shown as mean ± SEM. C) The dose response of ACh in depressing the synaptic responses is shown.

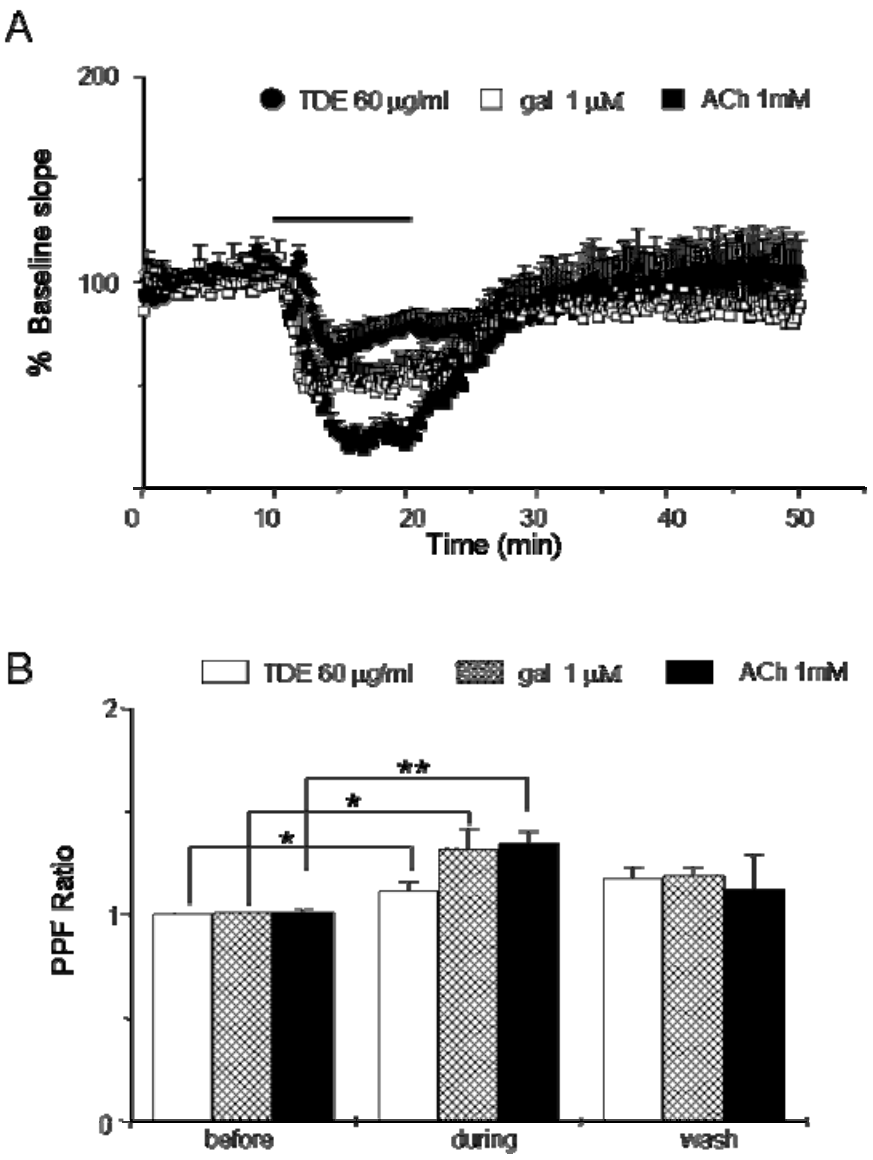

Figure. 2.5: The comparison of average depression of synaptic responses between 60 µg/ml of TDE, 1 µM galantamine (gal) and 1 mM of ACh. A) TDE- and galantamine-induced suppression of CA1 hippocampal synaptic responses appeared to be less and slower than ACh-induced suppression. B) TDE, galantamine and ACh significantly increased paired-pulse facilitation (PPF) (*: $p < 0.05$, **: $p < 0.01$ before comparing with during of TDE, galantamine or ACh application).

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กิตติกรรมประกาศ

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OUTPUT

การเสนอผลงานในงานประชุมทางวิชาการต่าง ๆ

1. **Chattipakorn SC**, Pongpanparadorn A, Pratchayasakul W, Pongchaidacha A, Ingkaninan K, Chattipakorn N. Tabernaemontana Divaricata extracts inhibit cholinesterase activity in rats brains. 2006:CINP. 109.

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2. Chattipakorn N, Shinlapawittayaorn K, Sungnoon R, **Chattipakorn S**. Fish oil does not improve defibrillation efficacy. *Int J Cardiol* 2007;120:289-96. Impact factor **2.234**

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บทความสำหรับการเผยแพร่

โรคอัลไซเมอร์ (Alzheimer's disease) เป็นโรคที่เกิดจากความผิดปกติของการทำงานของระบบประสาทในระบบประสาทโคลิเนอร์จิก (cholinergic system) โดยโรคนี้เป็นความผิดปกติของสมอง ที่มีอาการสมองเสื่อม ที่เรียกว่า dementia เกิดขึ้นโดยมีการถดถอยหน้าที่ของสมองผู้ป่วย จำเหตุการณ์และช่วงเวลาได้ไม่แน่นอน ความจำเสื่อม มีปฏิกิริยาโต้ตอบอย่างแปลกๆ พฤติกรรมเปลี่ยนแปลงไปในทางที่ไม่เหมาะสม อารมณ์จะไม่สม่ำเสมอ ซึ่งวิธีการรักษาโรคนี้ในปัจจุบันได้มุ่งเน้นไปที่การให้ยาหรือสารที่ไปออกฤทธิ์ยับยั้งการทำงานของเอนไซม์อะเซทิลโคลิเนสเทอ-เรส เพื่อเพิ่มระดับของสารสื่อประสาทอะเซทิลโคลีนในสมอง สำหรับยาที่ใช้ในการรักษาโรคอัลไซเมอร์ในปัจจุบันยังคงมีข้อเสียในเรื่องของการเกิดผลข้างเคียงต่างๆ เช่น ทำให้ คลื่นไส้ อาเจียน ท้องร่วง นอกจากนี้ยาที่มีใช้อยู่ในขณะนี้มีราคาแพงมาก ดังนั้น การศึกษาวิจัยหาสารหรือยาตัวใหม่สำหรับรักษาโรคนี้จึงยังคงเป็นที่น่าสนใจ เป็นที่รู้กันว่ายาแผนปัจจุบันที่ใช้ ในการรักษาหลายโรครวมทั้งรักษาโรคอัลไซเมอร์มีต้นกำเนิดมาจากพืชสมุนไพร เช่น ยากาเลนทามิน (galanthamine) ที่ใช้รักษาโรคอัลไซเมอร์ นั้น มีต้นกำเนิดมาจาก สมุนไพรจีนที่ชื่อว่า "snow drop" แล้วมีการศึกษาทดลองจนได้เป็นตัวยากาเลนทามินที่ใช้กันในปัจจุบัน สำหรับสมุนไพรไทยก็มีการใช้ในยาแผนไทยมากมาย ตัวยาตัวที่เป็นหนึ่งส่วนประกอบในสมุนไพรที่ใช้เป็นยาอายุวัฒนะไทยก็คือ ต้นพุดพิทยาหรือพุดซ้อน (*Tabernaemontana divaricata*) ซึ่งได้มีการศึกษาถึงคุณสมบัติของสารสกัดจากต้นพุดพิทยาหรือพุดซ้อนในหลอดทดลองก่อนหน้านี้พบว่า สารสกัดจากต้นพุดพิทยาหรือพุดซ้อน มีผลยับยั้งการทำงานของเอนไซม์อะเซทิลโคลิเนสเทอเรสได้สูงเช่นเดียวกับยากาเลนทามิน แต่ก็ยังไม่มีใครศึกษาถึงฤทธิ์ของสารสกัดจากต้นพุดพิทยาหรือพุดซ้อนในสัตว์ทดลองและในเซลล์ประสาทของสัตว์ทดลอง วัตถุประสงค์ในการศึกษาครั้งนี้เพื่อที่จะศึกษาผลของสารสกัดนี้ในการยับยั้งการทำงานของเอนไซม์อะเซทิลโคลิเนสเทอเรสในสัตว์ทดลองเปรียบเทียบกับการศึกษาในหลอดทดลองและศึกษาว่าถ้าสารสกัดจากต้นพุดพิทยา สามารถยับยั้งการทำงานของเอนไซม์อะเซทิลโคลิเนสเทอเรสได้ จะสามารถเพิ่มการทำงานของระบบประสาทในสมองได้หรือไม่ จากผลการศึกษาครั้งนี้แสดงให้เห็นว่า สารสกัดจากต้นพุดซ้อนมีผลในการยับยั้งการทำงานของเอนไซม์อะเซทิลโคลิเนสเทอเรสในสมอง นอกจากนี้ สารสกัดชนิดนี้ยังมีผลทำให้มีการเพิ่มการทำงานของระบบประสาทได้ในสมองของสัตว์ทดลอง และมีบทบาทในการควบคุมทำงานของการสื่อประสาทเช่นเดียวกับยากาเลนทามิน ดังนั้นสารสกัดจากต้นพุดพิทยานี้น่าจะมีประโยชน์ในการศึกษาหาตัวยาในสารสกัดจากต้นพุดพิทยาหรือพุดซ้อนใช้รักษาโรคอัลไซเมอร์ในอนาคตต่อไป